

AD-753 422

FURTHER CHARACTERIZATION AND SPECIES
SPECIFICITY OF LEUKOCYTIC ENDOGENOUS
MEDIATOR (LEM) (36842)

R. S. Pekarek, et al

Army Medical Research Institute of Infectious
Diseases
Frederick, Maryland

22 May 1972

DISTRIBUTED BY:

NTIS

National Technical Information Service
U. S. DEPARTMENT OF COMMERCE
5285 Port Royal Road, Springfield Va. 22151

Reported from: *Proc. Soc. Exp. Biol. and Med.* Volume 141, Number 2, November 1972
Copyright © 1972 by the Society for Experimental Biology and Medicine Printed in U.S.A.

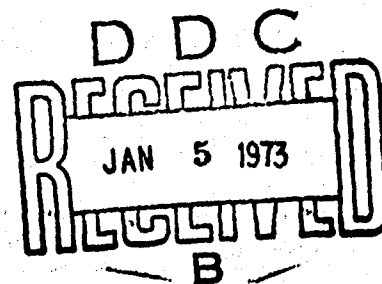
PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 141, 613-618 (1972)

**Further Characterization and Species Specificity of Leukocytic
Endogenous Mediator (LEM)¹ (36842)**

R. S. PEKAREK, R. W. WANNEMACHER, JR., F. E. CHAPPLE, III, M. C. POWANDA,

AD753422

Reproduction by
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. GOVERNMENT PRINTING OFFICE
WASHINGTON, D.C. 20540



Further Characterization and Species Specificity of Leukocytic Endogenous Mediator (LEM)¹ (36842)

R. S. PEKAREK, R. W. WANNEMACHER, JR., F. E. CHAPPLE, III, M. C. POWANDA,
AND W. R. BEISEL

U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Certain alterations in trace metal (1-6) and amino acid (4, 6, 7) metabolism during generalized infection and other inflammatory reactions appear to be mediated by an endogenous humoral factor released, in part, by polymorphonuclear (PMN) leukocytes. Leukocytic endogenous mediator (LEM) has been initially characterized as a heat labile, low-molecular weight (10,000-30,000) protein-like substance, which is soluble to some extent in organic solvents (3, 8). These properties are similar to those described for endogenous pyrogen (EP) (9), a low molecular weight protein which exhibits a tendency toward species specificity in its fever producing action (10) and can be inactivated with several proteolytic enzymes (11). Since neither LEM nor EP have been purified sufficiently to allow critical biological and chemical comparisons, it still remains uncertain whether they are one and the same, or different substances.

The purposes of the present study were to demonstrate the species specificity of LEM in terms of its effect on both trace metal and amino acid metabolism and to show the effects of various enzymes, pH changes and storage conditions on the activity of LEM. Further, since Kampschmidt and Upchurch (2) found that LEM prepared from rabbit PMN leukocytes could depress serum zinc in the rat and Wannemacher *et al.* (7) recently

demonstrated that acute serum from febrile patients infected with *Salmonella typhi* contained a factor which could alter both zinc and amino acid metabolism when administered to normal recipient rats, we investigated the potential value of employing rats as bioassay models for testing LEM preparations from other species.

Materials and Methods. Healthy male Dunning-Fisher rats weighing 150-175 g, adult male New Zealand white rabbits, young adult male rhesus monkeys, adult male dogs (mixed breeds), one young adult male goat, and one adult female burro were used in the studies. The isotope employed for studying amino acid flux was ¹⁴C-1-aminocyclopentane-1-carboxylic acid, cycloleucine (05.75 mCi/mmole, New England Nuclear, Boston, MA). Pronase, trypsin and lipase (Calbiochem, La Jolla, CA) were used in the enzyme studies.

Analytical methods. Serum zinc concentrations were determined by a previously described atomic absorption spectrophotometric method (12). Zinc-free polyethylene syringes, test tubes, and pipettes were used for sample collection and preparation to minimize the possibility of zinc contamination from exogenous sources. Liver homogenates were prepared and analyzed for the incorporation of the cycloleucine isotope by the method of Wannemacher *et al.* (7). The samples were counted in a 3-channel Nuclear Chicago scintillation counter with external standardization.

Species specificity of LEM. Crude preparations of LEM were obtained from rat, rabbit and monkey PMN leukocytes by a procedure described by Pekarek and Beisel (3). Protein concentrations of the different preparations were measured by the method of Lowry, *et al.* (13). Normal rats, rabbits and mon-

¹ In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

keys were placed into groups and administered either homologous or heterologous preparations of LEM as outlined in Table I. The recipient animals were bled at designated times (Table I), and their serum zinc concentrations were determined and compared to their respective control values. The rabbits and monkeys in each test group served as their own individual controls, whereas baseline normal serum zinc values in the rat were determined in corresponding saline inoculated controls, which were bled at the same time as the test groups were bled.

Assay of different LEMs in the rat. In addition to preparations from rat, rabbit, and monkey leukocytes, LEM release was also investigated in PMN leukocyte preparations of peritoneal exudates obtained from dogs, a goat, and a burro. All rats used in the assay system were fed an agar and gel diet, containing 18% casein (6), and 24 hr prior to use were injected subcutaneously with 1 μ Ci 100 g of body wt of cycloleucine. One half of the LEM preparation from each species was heat-inactivated (90° for 30 min). Both untreated LEM and heat-inactivated LEM from each species were then administered ip (1 ml) to respective groups of the pulse-labeled rats as outlined in Table II. Four hours after injection, the rats were bled and liver homogenates were obtained for the determination of their serum zinc and hepatic cycloleucine concentrations.

Effect of enzymes, pH and storage of LEM activity. LEM was prepared from PMN leukocytes obtained from rabbit peritoneal exudates. To 9 ml of LEM (3.8 mg protein/ml) 1 ml of either trypsin (3 mg) or lipase (4 mg) in 0.15 M tris(hydroxymethyl)-1-amino methane (pH 7.0) buffer, containing 4×10^{-3} M Ca^{2+} , was added, giving a final volume of 10 ml for each reaction mixture. Similarly, to another 9 ml of LEM a 1 ml of 0.10 M sodium borate buffer (pH 7.0) containing 4 mg of pronase and 5×10^{-3} M Ca^{2+} was added. The LEM-enzyme-buffer reaction mixtures were allowed to incubate at 37° for either 4 or 24 hr. After incubation 1 ml of each mixture was injected ip into normal cycloleucine labeled assay rats. As controls for this experiment, LEM-buffer

and enzyme-buffer mixtures were also administered in the same manner to respective groups of assay rats as shown in Table III. Four hours after administration of the various test mixtures from both the 4 and 24 hr incubation times, the rats were killed, and serum and liver samples were obtained for zinc and cycloleucine analysis.

To study the effect of pH on LEM activity, 1 ml of 0.5 M citric acid buffer, ranging from pH 4.5 to 8.0, was added to respective tubes containing 9 ml of LEM. The tubes were then placed at 4° overnight. After incubation the LEM-buffer mixtures were neutralized by the addition of 1 ml of 2 M sodium phosphate buffer, pH 7.0. The mixtures were given in 1 ml inocula ip to respective groups of cycloleucine labeled assay rats. The rats were killed 4 hr post administration and serum zinc and hepatic cycloleucine concentrations were determined and compared to saline controls.

To test the effects of storage on LEM activity, part of a freshly prepared lot of rabbit LEM (0.72 mg protein/ml) was administered (1 ml ip) to cycloleucine labeled rats and compared to saline controls for its effect on serum zinc depression and amino acid flux. The remaining LEM was divided into two lots and stored at 4 and -20°, respectively, for 7 days before being tested for activity (Table IV).

Results. As shown in Table I, a cross species susceptibility for the induction of serum zinc depressions was evident between LEM preparations obtained from and tested in rats, rabbits, and monkeys. However, based on the amount of LEM administered and the serum zinc depressions observed, both the rabbit and the rat appeared to be more susceptible than the monkey to homologous preparations of LEM. By contrast, the monkey appeared to be equally susceptible to the heterologous LEM preparations.

When alterations in serum zinc and hepatic cycloleucine concentrations in the rat were employed to assay the activity of LEM preparations obtained from peritoneal PMN leukocytes of a variety of species, all but the goat and burro LEM preparations were active, as shown in Table II. In all studies, the

TABLE I. Comparison of Species Specificity of LEM on Serum Zn Depression.

Source of LEM	Dose (mg protein)	Recipient animal		Control serum Zn* ($\mu\text{g}/100\text{ ml}$)
		Route of inoculation	Serum Zn* ($\mu\text{g}/100\text{ ml}$)	
Rabbit*				
Rabbit	1.98	iv	49 \pm 6 ^d	138 \pm 7
Rat	2.50	iv	73 \pm 7 ^d	133 \pm 5
Monkey	5.00	iv	86 \pm 3 ^d	126 \pm 7
Rat*				
Rabbit	0.66	ip	92 \pm 6 ^d	136 \pm 4
Rat	0.55	ip	53 \pm 4 ^d	144 \pm 4
Monkey	1.20	ip	89 \pm 4 ^d	138 \pm 5
Monkey*				
Rabbit	10.00	iv	55 \pm 3 ^d	97 \pm 8
Rat	7.50	iv	70 \pm 4 ^d	91 \pm 5
Monkey	12.00	iv	50 \pm 5 ^d	101 \pm 6

* Minimum of 6 determinations/value \pm SE.

* Measured at 12 hr postadministration.

* Measured at 6 hr postadministration.

* Values significantly different from control $p < .01$.

heat-treated preparations were inactivated, and thus did not induce any changes in either of the two parameters being measured.

The two proteolytic enzymes, trypsin and pronase, were only partially effective in inactivating LEM when incubated for 4 hr (Ta-

ble III). However, both enzymes were equally effective in inactivating the ability of LEM to induce either serum zinc depression or amino acid flux when the LEM-enzyme-buffer reaction mixtures were allowed to incubate for 20 hr. As shown in Table III, lipase

TABLE II. Effect of LEM Prepared From Different Species on Serum Zinc Depression and Amino Acid Flux in the Rat.

Species	Protein content (mg/ml)	Treatment	Hepatic* cycloleucine (dpm/mg)	Serum* zinc ($\mu\text{g}/100\text{ ml}$)
Rat	0.14	Untreated	61.6 \pm 2.0 ^a	74 \pm 5 ^b
		Heat inactivated	33.6 \pm 1.6	122 \pm 4
Rabbit	1.50	Untreated	42.1 \pm 2.4 ^a	50 \pm 3 ^b
		Heat inactivated	30.1 \pm 1.3	133 \pm 5
Monkey	1.30	Untreated	26.2 \pm 1.2 ^a	64 \pm 3 ^b
		Heat inactivated	17.9 \pm 0.9	120 \pm 7
Dog	4.20	Untreated	36.5 \pm 2.9 ^a	57 \pm 4 ^b
		Heat inactivated	20.0 \pm 0.8	118 \pm 5
Goat	1.50	Untreated	21.8 \pm 1.2	126 \pm 5
		Heat inactivated	14.8 \pm 2.1	103 \pm 7
Burro	3.60	Untreated	23.4 \pm 1.4	118 \pm 8
		Heat inactivated	26.1 \pm 3.2	120 \pm 6

* Means of a minimum of 6 animals \pm SE.* Values significantly different from control $p < .01$.

TABLE III. Effect of Pronase, Trypsin and Lipase on Amino Acid Flux and Serum Zinc Depression Activity of Rabbit LEM When Tested in the Rat

Treatment	Hours of incubation			
	4		20	
	Cycloleucine* (dpm/mg liver)	Serum zinc* (μ g/100 ml)	Cycloleucine* (dpm/mg liver)	Serum zinc* (μ g/100 ml)
Buffer + pronase	25.6 \pm 1.2	138 \pm 8	26.6 \pm 2.9	111 \pm 7
LEM + buffer + pronase	33.7 \pm 2.5	86 \pm 14	26.7 \pm 2.4	122 \pm 8
LEM + buffer	40.3 \pm 1.9	19 \pm 5	66.4 \pm 2.0	58 \pm 6
Buffer + trypsin	26.8 \pm 1.8	141 \pm 2	28.2 \pm 0.9	150 \pm 6
LEM + buffer + trypsin	34.8 \pm 3.0	72 \pm 9	33.2 \pm 2.8	135 \pm 7
LEM + buffer	45.8 \pm 2.8	49 \pm 6	68.0 \pm 3.9	45 \pm 4
Buffer + lipase	—	—	26.3 \pm 1.2	153 \pm 4
LEM + buffer + lipase	—	—	53.1 \pm 4.0	86 \pm 4
LEM + buffer	—	—	66.8 \pm 5.0	40 \pm 4

* Means of a minimum of 6 animals \pm SE.

was able to partially reduce the activity of LEM. Since the lipase pretreatment elicited some degree of inactivation, the lipase preparation was tested for proteolytic activity (14) and was shown to be about 1/20 as active (on a wt basis) as the trypsin used in this study.

Pretreatment of LEM with buffers ranging from pH 4.5-8.0 showed little effect on reducing the activity of LEM, especially for amino acid flux, as shown in Fig. 1. However, slight variations in activity were observed for serum zinc depression. Storage at 4° for 7 days did not alter the activity of LEM for either parameter measured. By contrast, a

slight loss of activity for serum zinc depression but not for amino acid flux was observed when the LEM was stored at 20° for 7 days.

Discussion. The effects of LEM obtained from PMN leukocytes on serum zinc and iron concentrations (1-3) and amino acid metabolism (4, 6, 7), have been clearly demonstrated when measuring these parameters in rat and rabbit models. However since LEM shares several characteristics with EP studies on the species specificity and various chemical and physical properties of LEM were conducted in order to establish additional similarities or differences between these two endogenous mediating factors of the inflammatory process.

The present study demonstrated that a cross species susceptibility to heterologous LEM preparation existed between the rat, rabbit and monkey; with the monkey being equally susceptible to both the heterologous and homologous preparations. The responses observed for LEM between the rat and rabbit for serum zinc depression and amino acid metabolism differed somewhat from those reported for EP. Both leukocytic extracts from the rat and rabbit could induce alterations in zinc and amino acid metabolism when tested in either species, whereas Kampschmidt and Upchurch (1) found that leukocytic extract from rabbit PMN leukocytes increased the

TABLE IV. Effect of Storage on the Amino Acid Flux of Serum Zinc Depression Activity of LEM in the Rat.

Treatment	Hepatic cycloleucine* (dpm/mg)	Serum zinc* (μ g/100 ml)
Saline control	19.1 \pm 1.8	125 \pm 8
LEM zero time	28.7 \pm 2.1 ^b	56 \pm 6 ^b
LEM 4° for 7 days	31.5 \pm 3.8 ^b	59 \pm 7 ^b
LEM -20° for 7 days	33.6 \pm 3.6 ^b	87 \pm 8 ^c

* Means of a minimum of 6 animals \pm SE.^b Value significantly different from control: $p < .01$; ^c $p < .05$.

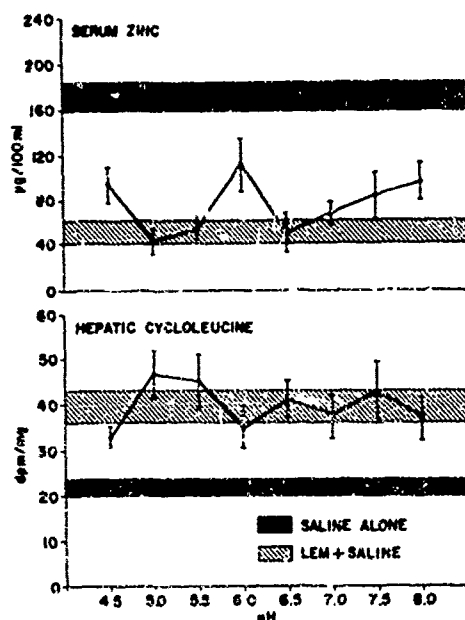


FIG. 1. The effect of varying pH on the serum zinc depression and amino acid flux activity of LEM. The solid horizontal bands represent the means \pm SE of saline controls, and the shaded horizontal bands represent the means \pm SE of unmodified LEM plus saline.

body temperature of rabbits, but decreased and altered the temperature cycle in the rat. Furthermore, these investigators reported (1) that leukocytic extracts from rat PMN leukocytes had no effect upon the body temperatures of either the rat or rabbit. Since the liver appeared to be the site of action for the lowering of serum zinc (5) and amino acid flux (6) and the hypothalamic area the site of thermoregulation (9) the differences in the observed responses could possibly be due to the differences in either the effector sites between species or to differences between the effector molecules (LEM and EP) themselves.

Although the monkey appeared to be equally susceptible to rat and rabbit LEM its employment as a model for the routine bioassay of LEM is impractical. Therefore the rat was employed, and, as demonstrated by this study, depressions in serum zinc and increases in hepatic cycloleucine concentrations could be induced by LEM from all

species tested with the exception of the goat and burro. Although these findings may appear to limit the rat as a bioassay animal to certain species, recent studies (4, 7) have shown that sterile "acute" serum from infected humans could induce both serum zinc depressions and an amino acid flux when administered to the rat; a finding that has potential usefulness for studying the pathogenesis of inflammatory diseases in man.

The results from the enzyme studies confirm the fact that LEM, like EP, is composed of protein. However, LEM appeared to be more resistant than EP to the action of proteolytic enzymes. Rafter, Collins and Wood (11) reported that EP could be inactivated after only 4 hr of treatment with trypsin (3 mg/11 ml) and within 2 hr with pepsin (2 mg/10 ml). By contrast a 4-hr incubation with either trypsin (3 mg/10 ml) or pronase (4 mg/10 ml) only partially inactivated the serum zinc depressing and amino acid flux effects of LEM. Only after a 20-hr incubation with either of these two proteolytic enzymes was LEM inactivated, suggesting that it may have a different amino acid sequence or structural configuration, which renders it more resistant to proteolytic enzymes.

LEM appears to be relatively stable over a wide pH range; although slightly more variability on the zinc depressing effects were observed. Similarly storage of LEM at 4° did not appear to reduce its potency. However, the serum zinc depressing effects were somewhat reduced when the LEM was stored at -20° . These slight but obvious differences on the effects of LEM on serum zinc depression and amino acid flux after varying the pH or storing at -20° may suggest that the two biochemical parameters measured in this study may, in themselves, be mediated by separate proteins released, in part, by PMN leukocytes.

Although LEM and EP are both derived from PMN leukocytes and share certain physical and chemical properties, differences in these properties and species specificity exist. These inconsistencies can only be explained by the ultimate purification of these endogenous factors.

Summary. A degree of cross species suscep-

tibility was observed on the effects of leukocytic endogenous mediator (LEM) on serum zinc depression and amino acid flux. LEM was shown to be protein in nature, and relatively stable under a wider range of pH and conditions of storage. Although LEM shares several chemical and physical characteristics with endogenous pyrogen (EP), some differences between the chemical properties and species specificity of these two mediators were noted.

1. Kampschmidt, R. F., and Upchurch, H. F., *Amer. J. Physiol.* 216, 1287 (1969).
2. Kampschmidt, R. F., and Upchurch, H. F., *Proc. Soc. Exp. Biol. Med.* 134, 1150 (1970).
3. Pekarek, R. S., and Beisel, W. R., *Proc. Soc. Exp. Biol. Med.* 138, 728 (1971).
4. Pekarek, R. S., Commission on Epidemiological Survey Annual Report FY 1971, p. 99 (1971).
5. Pekarek, R. S., Wannemacher, R. W., Jr., and Beisel, W. R., *Proc. Soc. Exp. Biol. Med.* 140, 685 (1972).
6. Wannemacher, R. W., Jr., Pekarek, R. S., and Beisel, W. R., *Proc. Soc. Exp. Biol. Med.* 139, 128 (1972).
7. Wannemacher, R. W., Jr., Pekarek, R. S., Powanda, M. C., Beisel, W. R., DuPont, H. L., Schwartz, A., and Hornick, R. B., *J. Infect. Dis.* 126, 77 (1972).
8. Kampschmidt, R. F., and Upchurch, H. F., *Proc. Soc. Exp. Biol. Med.* 133, 128 (1969).
9. Atkins, E., and Bodel, P., *N. Engl. J. Med.* 286, 27 (1972).
10. Bornstein, D. I., and Woods, J. W., *J. Exp. Med.* 130, 707 (1969).
11. Rafter, G. W., Collins, R. D., and Wood, W. B., Jr., *J. Exp. Med.* 111, 831 (1960).
12. Pekarek, R. S., and Beisel, W. R., *Appl. Microbiol.* 18, 482 (1969).
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.* 193, 265 (1951).
14. Rinderknecht, H., Geokas, M. C., Silverman, P., and Haverback, B. J., *Clin. Chim. Acta* 21, 197 (1968).

Received May 22, 1972. P.S.E.B.M., 1972, Vol. 141.